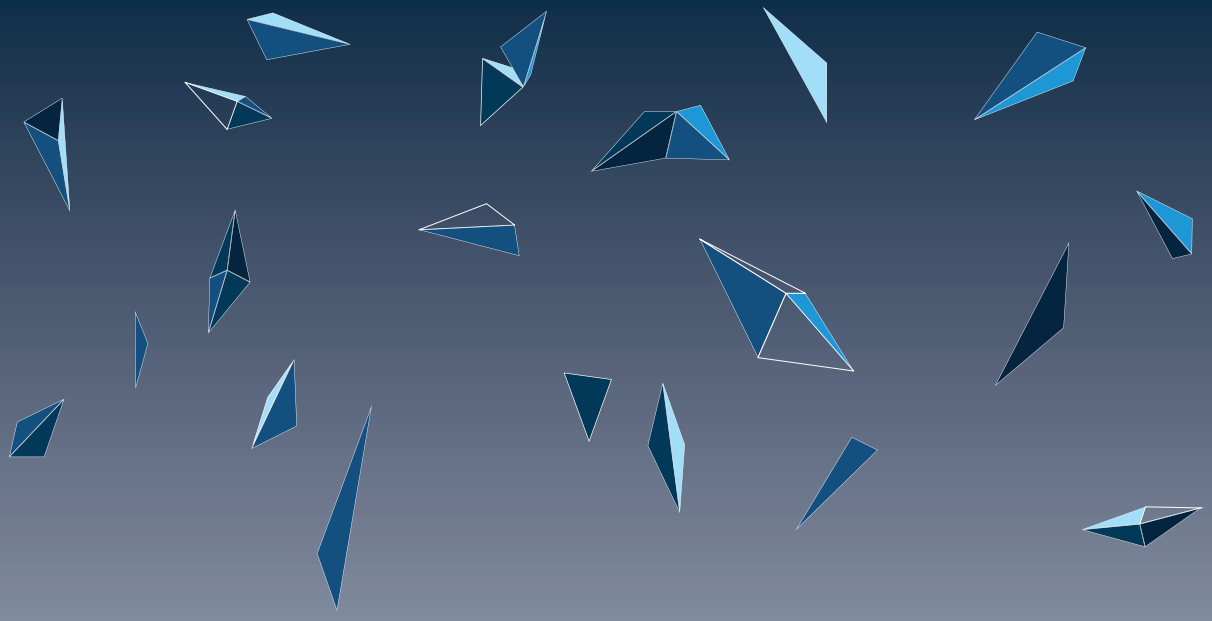
Early differentiation dynamics of the trophoblastic lineage and its cross-communication with the embryo



Chapter 7.
Discussion and valorization of results

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In this chapter we intend to evaluate the findings described in this thesis and put them in a perspective of potential applications in the fields of stem cell biology, developmental biology and the new field of synthetic embryology.

Cutlure of TSC

The traditional culture of TSC makes the use of a feeder layer of cells serum, which often represent an obstacle in the development of new models. This is because these two factors provide a large supply of unknown molecules that might be detrimental for some of the properties we may want to exploit form a particular cell type. The use of feeder layer and serum, also means a potential variability across laboratories due to different batches, that prevent full control of the culture conditions and reproducibility. These two limitations of the classical culture can now be avoided thanks to the development of new conditions such as the Tx media. Although this media eluded the use of serum or feeder cells, it leads to a culture with a high degree of inter-cellular heterogeneity that we hypothesize to represent of spectrum of differentiation states observed along the TE axis and beyond. This same observation was already made in the culture of ESC, the embryonic equivalent of the TSC, a problem that was solved by the development of the so known 2i media.
Such inter-cellular heterogeneity we observe allows us to confirm the role of Cdx2 as a gene controlling the undifferentiated state of TSC. We found Cdx2 expression to be directly correlated with stem cell related properties such as self-renewal and proliferation, making it an ideal gene to act as readout to improve the culture conditions. On the other hand, this heterogeneous culture results in a spectrum of differentiation states limiting the applications of TSC.

Screenings for improving TSC culture

Screenings are an extremely valuable tool in many fields of life sciences, but in cell and developmental biology play a particularly important role, for example in primary cell line derivation. Although researchers generally have candidate factors to add on the culture media when deriving a new cell line (based for example on information acquired from observations made on mutant mice), traditionally several conditions are tested aiming to obtain a cell type showing a phenotype similar to that observed in the cells in vivo. These screenings are relatively simple and rely on testing one compound at the time and are sufficient for a qualitative result: whether the cell type of interest is derived in vitro or not. However, when we use screenings with a quantitative readout aiming to maximize the expression of a particular gene, factorial design becomes a very useful tool, as long as there are multiple single compounds having the desired effect. In our case, several modulators of different pathways were considered as screening "hits" based on our readout (increased Cdx2 expression). Several factors such as feedback loops or pathway crosstalk may play a role when multiple pathways interact, potentially leading to negative effects such as cellular differentiation or toxicity, or in contrary, they could have a cooperative effect. Factorial design of combinatorial screenings help us combine those initial hits and figure out which of the single compounds have a more beneficial effect while preventing us from performing experimentally all the potential combinations, but they also serve as tool for discovering compound or pathway interactions.
From the factorial design we tested 21 cocktails, with 4 of them reaching elevated and stable Cdx2 expression levels but showing different cells morphologies with one of them resulting in the phenotype we aimed for.

In the field of human ESC, there has been a constant search for new culture conditions that allow for culturing the cells in a more naïve state. In a previous publication aiming to upregulate Oct4 expression on hESCs (Gafni et al., 2015), several positive regulators were obtained that were first combined and then subdivided into two pools that were tested separately. Although the cocktail resulted in upregulated in Oct4 expression, with this strategy it is unclear if such expression could have been increased even further, possibly resulting in a different phenotype. We believe factorial design to be underrepresented tool in the optimization of in vitro models, for us it played a pivotal role in the findings described in this book.

The blastoid

This in vitro model for the mouse pre-implantation embryo combines ESCs and TSCs that under specific culture conditions cooperate in order to form structures that morphologically and functionally resemble the E3.5 blastocysts. Interestingly, during the blastoid protocol, the cultured cells switch to a phenotype much more comparable to the one observed of the cells in the embryo, and they help each other maintain an undifferentiated state. We can witness a permanent cross-communication between both compartments and we have successfully delineated it by performing lineage specific knockouts. Importantly, the blastoids are also capable of implanting in the uterus of pseudo-pregnant females, triggering a response in the endometrium only comparable to the one obtained by a blastocyst. However, the development in utero only progresses for one or two days after implantation.

There are some aspects of the blastoid protocol that remain to be improved. One of those is obtaining a more efficient second lineage commitment within the ICM. With the current protocol, this event takes place, however it happens at a low rate. Forming blastoids is highly dependent on the culture of TSCs, which we consider the main factor affecting the blastoid formation efficiency, making the TSCs pre-culture a second factor to solve. Some TSC lines are incapable of cooperating with ESC to form blastoids, and we hypothesize this to be a result of those TSCs not being representative of the blastocyst TE. Obtaining a culture of TSC that shows a higher similarity with the cells in the TE would potentially lead to higher blastoid formation efficiencies and an enhanced cross-communication with the embryonic compartment. Both primitive endoderm formation and a more robust trophoblast epithelium need to be achieved in the blastoid before we consider the blastoid to progress further after implantation.

Potential improvements aside, the blastoid is already a valuable tool for a variety of applications in the fields of developmental and reproduction biology. Blastoids might become an instrumental tool for toxicological and genetic screenings, and if someday a blastoid is capable to lead to live pups, it will make individual cloning possible.

Polar like TSCs in blastoid formation

Combinatorial screenings allowed us to obtain a new culture cocktail with increased Cdx2 expression levels in a stable manner. After further optimization by switching to a fully chemically-defined plate coating and a compound concentration reduction we obtained a culture we call Lt21, that lead to polar-like TSCs (plTSCs) with enhanced stem cell properties and that allows us for direct derivation of new lines.

Importantly cells cultured in these new conditions reach the expectation of being more efficient at forming blastoids both when the cells cultured in TX are converted to polar-like conditions and when they are directly derived in them. In order to be considered a blastoid, a structure must include both cell types with the ESCs engulfed by the TSCs, form a cavity

and reach certain size and circularity. Both blastoids and trophospheres obtained from the use of plTSCs show an increased circularity suggesting this to be an intrinsic property of the plTSCs, but only blastoids show an increased diameter by swelling, which suggests a more efficient crosstalk with the ICM. plTSCs are also more prone to cavitation since they are not as dependent on ESCs to trigger this process. Blastoids obtained from plTSCs cells show correct distribution of compartments and allow for ESCs differentiation into PrE-like cells. These new culture conditions show clear improvements when it comes to improving the efficiency of the blastoid protocol without affecting any other parameters, and we hypothesize this to be a result of an improved epithelial phenotype, a improved expression of the core transcription factors, and a better cross-talk with the ESCs, possibly by being in a more developmentally equivalent state, hence the name polar-like TSC.

Polar-like TSC reduced heterogeneity

As shown on chapter 1, TSCs cultured in Tx conditions display a notorious inter-cellular heterogeneity with cells being present in a partially differentiated state. Although a validated model for properly quantifying culture heterogeneity is lacking, our shows that the LT21 culture conditions partially correct for such heterogeneity, especially when the cell line is directly derived under polar like conditions. That heterogeneity is greatly reduced when it comes to expression of Cdx2 protein, but this also happens in wider terms as suggested by our transcriptome and phenome results.
Transcriptome analysis also allows us to find markers of differentiated and undifferentiated states, such as Ly6a, which we have proven to be preferentially expressed in the polar TE of the blastocysts.

TE axis formation

Over the years many researchers have reported a different, partly reversible phenotype between polar and mural trophectoderm (Chavez, Enders, & Schlafke, 1984; Copp, 1978; Cruz & Pedersen, 1985; Gardner, 2000). Although this different identity has mainly been observed in the late blastocysts (E4.5) we have tried to assess this in the 3.5 blastocysts. For this we have primarily used two tools: blastocyst tomo-sequencing and blastocyst whole mount fluorescent in situ hybridization (smFISH). Each of these techniques has its advantages and its limitations. Tomo-sequencing is a technically difficult experiment to perform due to the size of the tissue and the need for a near perfect orientation upon mounting. However, it allows to obtain genome-wide results without requiring initial candidates. However, the protocol we followed by RNA extraction relies on the presence of PolyA sequences on the mRNA, which are not present in all genes. Bearing this in mind, we are unable to make interpretations involving a large number of genes, including Cdx2 or Nanog. Also, similar to the case of single cell sequencing, we are unable to reliably detect lowly expressed genes such as transcription factors. It could be, nevertheless an extremely useful technique when the goal is to find new markers following a particularly expression pattern. smFISH on the other hand has the limitations of requiring a candidate gene and can be performed for one to three genes at a time only. However, it is a far more sensitive technique when it comes to detect a higher percentage of the transcripts of the gene of interest and allows us to spatially locate them. Combining these two techniques is instrumental for finding new unknown markers.
Although we do see differential gene expression patterns from the results obtained in the blas-

tocyst, we believe that these divergent phenotypes between mural and polar trophectoderm might become more evident in the late blastocyst. Performing multiple tomo-sequencing experiments at different blastocyst stages would allow us to better characterize and therefore understand those divergent fates.

Transcriptomics data from trophoblasts obtained from blastoids show a high heterogeneity when those blastoids have been made from plTSCs, suggesting an efficient axis formation. smFISH polarity data confirms the symmetry breaking to occur in blastoids from plTSCs. For one of the two genes tested (Cdx2), the frequency at which a preferential polar distribution occurs is higher than that one observed in the blastocyst. This could suggest that the TE of blastoids obtained with plTSCs are no longer equivalent to a 3.5 blastocyst but to a later stage.